

## Regulation of Glycosaminoglycan Synthesis by Thyroid Hormone in Vitro

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### Research Article

Human skin fibroblasts synthesize and accumulate glycosaminoglycans (GAG). Recently, we reported that fibroblasts incubated in thyroid hormone-deficient media accumulate more GAG than do cultures incubated in the same media enriched with 0.1  $\mu\text{M}$  triiodothyronine ( $\text{T}_3$ ) (1981. *Endocrinology*. **108**: 2397). The current study characterizes that enhanced accumulation. Confluent cultures were maintained in thyroid hormone-deficient media without or with added  $\text{T}_3$ , labeled with [ $^3\text{H}$ ]acetate and analyzed for total [ $^3\text{H}$ ]GAG and [ $^3\text{H}$ ]hyaluronic acid content.

Addition of  $\text{T}_3$  to thyroid hormone-depleted media consistently inhibited the incorporation of [ $^3\text{H}$ ]acetate into GAG by 28-60% in fibroblast cultures from four different normal human donors. Maximal inhibitory effect was observed within 3 d after hormone addition at concentrations  $> 1$  nM. 73% of the maximal inhibitory effect was observed in the presence of physiologic concentrations of  $\text{T}_3$  (0.16 nM total  $\text{T}_3$  or 1.4 pM free  $\text{T}_3$ ).

The following observations indicated that  $\text{T}_3$  inhibition of [ $^3\text{H}$ ]GAG accumulation is most likely due to a decrease in GAG synthesis rather than to changes in the acetate pool or GAG degradation: (a) Addition of 0, 100, 500, and 2,500  $\mu\text{M}$  unlabeled acetate progressively decreased [ $^3\text{H}$ ]acetate incorporation into GAG, up to 80%, without altering the further inhibitory effect of  $\text{T}_3$  (35-40%); (b). A similar effect of  $\text{T}_3$  on GAG (32% inhibition) was observed using [ $^3\text{H}$ ]glucosamine as substrate; (c)  $\text{T}_3$  decreased hyaluronate synthetase activity [...]

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# Regulation of Glycosaminoglycan Synthesis by Thyroid Hormone in Vitro

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**ABSTRACT** Human skin fibroblasts synthesize and accumulate glycosaminoglycans (GAG). Recently, we reported that fibroblasts incubated in thyroid hormone-deficient media accumulate more GAG than do cultures incubated in the same media enriched with 0.1  $\mu$ M triiodothyronine ( $T_3$ ) (1981. *Endocrinology*. 108: 2397). The current study characterizes that enhanced accumulation. Confluent cultures were maintained in thyroid hormone-deficient media without or with added  $T_3$ , labeled with [ $^3$ H]acetate and analyzed for total [ $^3$ H]GAG and [ $^3$ H]hyaluronic acid content.

Addition of  $T_3$  to thyroid hormone-depleted media consistently inhibited the incorporation of [ $^3$ H]acetate into GAG by 28–60% in fibroblast cultures from four different normal human donors. Maximal inhibitory effect was observed within 3 d after hormone addition at concentrations  $> 1$  nM. 73% of the maximal inhibitory effect was observed in the presence of physiologic concentrations of  $T_3$  (0.16 nM total  $T_3$  or 1.4 pM free  $T_3$ ).

The following observations indicated that  $T_3$  inhibition of [ $^3$ H]GAG accumulation is most likely due to a decrease in GAG synthesis rather than to changes in the acetate pool or GAG degradation: (a) Addition of 0, 100, 500, and 2,500  $\mu$ M unlabeled acetate progressively decreased [ $^3$ H]acetate incorporation into GAG, up to 80%, without altering the further inhibitory effect of  $T_3$  (35–40%); (b) A similar effect of  $T_3$  on GAG (32% inhibition) was observed using [ $^3$ H]glucosamine as substrate; (c)  $T_3$  decreased hyaluronate synthetase activity by 32%; and (d) There was no effect of  $T_3$  on GAG degradation in a pulse-chase experiment. The

effect of  $T_3$  on [ $^3$ H]GAG accumulation appears to be quite specific, since the hormone had no effect on the incorporation of [ $^3$ H]leucine into trichloroacetic acid-precipitable material.

Thus, thyroid hormone inhibits GAG accumulation in a dose-, time-dependent, and reversible manner. This inhibition is apparently due to specific effects on the rate of macromolecular synthesis.

## INTRODUCTION

Thyroid hormone modulates many aspects of cellular metabolism (1, 2). In man, hypothyroidism is characterized by a constellation of clinical signs and symptoms, including growth failure and mental retardation (3). In severe, long-standing hypothyroidism, there is a tendency for the deposition of mucinous material in skin, resulting in a characteristic nonpitting edema. This material is composed of acid mucopolysaccharides (glycosaminoglycans, GAG),<sup>1</sup> complex polysaccharides normally found in small amounts (4).

Human skin fibroblasts offer the experimentalist an easily accessible tissue that can be propagated in culture and retain many of the phenotypic expressions typical of differentiated cells. Several biological aspects of the skin fibroblast have been studied in recent years. They are known to retain hormone receptors for triiodothyronine, dexamethasone, insulin, and androgen (5–8). Responses to several hormones including glucocorticoids, prostaglandins, catecholamines, and insulin have been demonstrated in fibroblasts (9–13).

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<sup>1</sup> *Abbreviations used in this paper:* BS, bovine serum; D-FCS, thyroid hormone-depleted FCS; D-FCS +  $T_3$ , D-FCS to which  $T_3$  was added; FCS, fetal calf serum; GAG, glycosaminoglycans; HA, hyaluronic acid;  $T_3$ , triiodothyronine;  $T_4$ , tetraiodothyronine or thyroxine; Tx-BS, BS from thyroidectomized animals; Tx-BS +  $T_3$ , Tx-BS with added  $T_3$ ; UDP-GlcNAc, uridine diphosphate-N-acetyl glucosamine; UDP-GlcUA, uridine diphosphate glucuronic acid.

Little is known about the effect of thyroid hormone in human skin fibroblasts. Current information can be summarized as follows: (a) The two metabolically active thyroid hormones, thyroxine ( $T_4$ ) and triiodothyronine ( $T_3$ ) are transferred from medium to cells. This cellular uptake of hormone is directly proportional to the concentration of free  $T_4$  and  $T_3$  in the medium (14); (b) As in other tissues, metabolism of thyroid hormones by fibroblasts involves a conversion of  $T_4$  to  $T_3$  (14); (c) Fibroblasts possess high affinity nuclear binding sites for  $T_3$  (5, 15); and (d) Observed biologic effects of thyroid hormone include stimulation of growth (16), glucose utilization (14, 17), lactate production (17), and degradation of low density lipoproteins (18).

Recently, we reported that thyroid hormone inhibits the accumulation of GAG in a human skin fibroblast culture incubated in a thyroid hormone-deficient medium (19). The current report describes subsequent studies in fibroblast cultures from several individuals, which were undertaken to determine the kinetics, specificity, reversibility, and dose dependence of this inhibition, as well as to ascertain whether the accumulation of GAG was the result of a changed rate of synthesis or degradation.

## METHODS

### Materials

$[^3H]$ Acetic acid (2,000 mCi/mmol sp act) and uridine diphosphate- $[^{14}C]$ glucuronic acid (UDP- $[^{14}C]$ GlucUA) (343 mCi/mmol sp act),  $[^3H]$ glucosamine (20,300 mCi/mmol sp act) and L- $[^3H]$ leucine (1,110 mCi/mg) were obtained from New England Nuclear, Boston, MA. Streptomyces hyaluronidase was obtained from Miles Laboratories, Inc., Elkhart, IN. All chemicals used were of the highest purity commercially available. Uridine diphosphate sugars were chromatographed on two thin-layer systems to determine purity.

### Procedures

**Preparation of thyroid hormone-depleted sera.** A modification of the method of Samuels et al. (20) was used to remove thyroid hormone from fetal calf serum (FCS). Rexyn 201 or 202 ion-exchange resins (Fisher Chemical Co., Pittsburgh, PA) were washed extensively and were used interchangeably. Resins were incubated with FCS at room temperature on a gyratory shaker. Later experiments utilized bovine serum from a thyroidectomized animal (Tx-BS). The concentrations of total  $T_4$  and  $T_3$  and free (dialyzable)  $T_3$  in various sera are given in Table I. Media supplemented with 10% FCS and BS contained 0.25 and 0.17 nM  $T_3$  respectively, compared with 0.063 and 0.068 nM  $T_3$  for media supplemented with 10% D-FCS and Tx-Bs, respectively. Thus, addition of 0.1 nM  $T_3$  to a medium containing thyroid hormone-depleted serum (D-FCS and Tx-BS) brought the serum total  $T_3$  concentrations to 0.163 and 0.168 nM, and the free  $T_3$  concentrations to 1.39 and 1.01 pM respectively, or to almost physiologic levels. All sera were sterilized

TABLE I  
Thyroid Hormone Concentration of Sera

Source of serum	Total $T_4$		Total $T_3$		Free $T_3$	
	$\mu\text{g/dl}$	$\text{ng/dl}$	nM	%	pM	
FCS	14.5	164	2.52	0.083	2.09	
BS	5.1	111	1.71	0.096	1.64	
Human serum	8.1	130	2.00	0.100	2.00	
D-FCS	<0.2	41	0.63	0.085	0.54	
Tx-BS	<0.2	44	0.68	0.060	0.41	

by Millipore (0.22  $\mu\text{m}$ ) filtration (Millipore, Corp, Bedford, MA) before addition to the culture medium.

**Cell culture.** Human skin fibroblasts obtained from several subjects by skin punch biopsy, were used between the 6th and 11th serial passages beyond the initial plating. For experiments, cultures were grown to confluence in 60-mm Diam plastic petri dishes using a modified Dulbecco's medium with Earle's salts, 50  $\mu\text{g/ml}$  ascorbic acid, 1  $\mu\text{g/ml}$  ferric nitrate, and enriched with 10% FCS at 37°C in an atmosphere containing 10%  $\text{CO}_2$  and a relative humidity of 100%. Glutamine 435  $\mu\text{g/ml}$  and kanamycin 100  $\mu\text{g/ml}$  were added.

Experiments were carried out only after cultures had reached confluence. Cells were then exposed to media containing either normal serum (FCS or BS), thyroid hormone-depleted serum (D-FCS or Tx-BS) or thyroid hormone-depleted serum supplemented with  $T_3$  (D-FCS +  $T_3$  or Tx-BS +  $T_3$ ) and further incubated for the indicated periods of time.

For  $[^3H]$ GAG accumulation studies, media were removed 24 h before harvesting and substituted with fresh media containing the same serum and hormone additives with either 10  $\mu\text{Ci/ml}$  of  $[^3H]$ acetate (total 40  $\mu\text{Ci/plate}$ ) or 5  $\mu\text{Ci/ml}$   $[^3H]$ glucosamine (total 20  $\mu\text{Ci/plate}$ ). Various amounts of unlabeled sodium acetate were added to the media in order to examine the influence of the saturable uptake of acetate on the  $T_3$ -mediated changes of  $[^3H]$ GAG accumulation.  $[^3H]$ Leucine (5  $\mu\text{Ci/ml}$ ) was added to the culture medium 24 h before harvesting in an experiment that examined the effect of  $T_3$  on protein synthesis. Finally, in the pulse-chase experiment, 60  $\mu\text{Ci}$  of  $[^3H]$ acetate were added in 4 ml of medium per plate and the labeling time was shortened to 12 h. Following three washes of the cell layers with 10 ml serumless medium, 4 ml of the corresponding serum-containing media, devoid of isotope but with 1 mM unlabeled acetate, was added and incubation carried out for various times over 63 h.

**Quantification of  $[^3H]$ GAG accumulation.** After labeling, the medium was removed, the cell layer was washed with serumless medium and then disrupted by sonication (sonifier, model 183, Branson Sonic Power Co., Danbury, CT) in 3 ml of 0.1 N NaOH. After removal of an aliquot of the cellular material for protein determination (21), the cell layer and medium were combined, neutralized with HCl, and digested with 1 mg/ml pronase in 100 mM Tris buffer pH 8.0 at 50°C for 16 h, in the presence of 250  $\mu\text{g}$  of both hyaluronic acid (HA) and chondroitin sulfate, which were added as carriers. After cooling on ice, trichloroacetic acid (TCA) was added to a final concentration of 5% and the samples were allowed to precipitate for 1 h. After centrifugation at 10,000 g for 10 min, the nonprecipitable material was dialyzed extensively against water at 4°C and then ly-

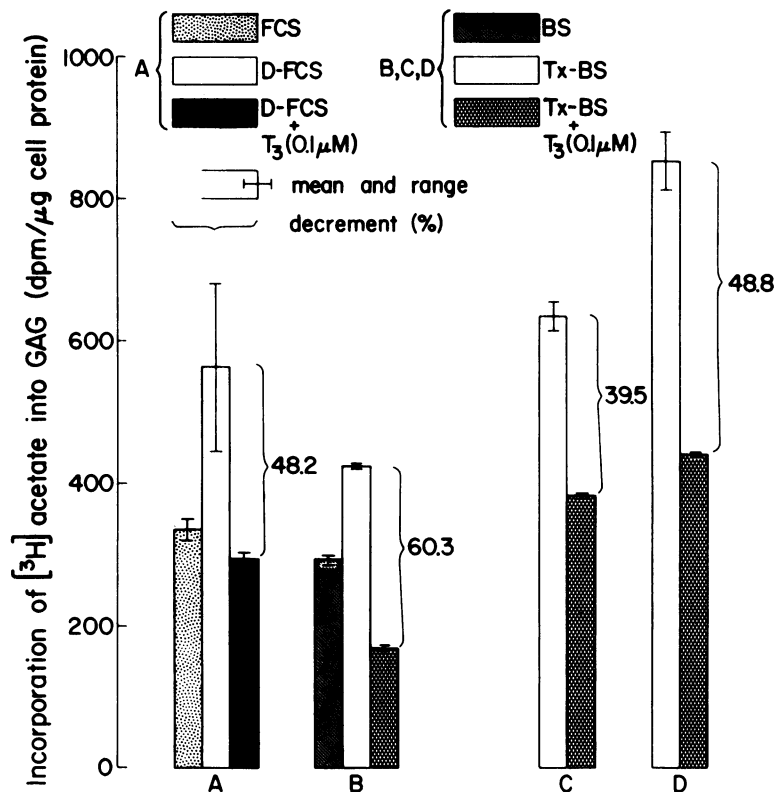


FIGURE 1 Reproducibility of the effect of  $T_3$  on the incorporation of  $[^3H]$ acetate into GAG. All experiments were conducted on fibroblasts obtained from the same subject, and grown to confluence in media containing 10% FCS. The medium was then substituted as indicated in the figure key, for 3 d before harvest. Experiments A and B were carried out simultaneously, while experiments B, C, and D were conducted at different times over a 5-mo period.

ophilized to dryness. The residue was resuspended in 0.15 M NaCl. A small aliquot was counted in a Packard TriCarb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) and defined as total  $[^3H]GAG$ .<sup>2</sup> Results are expressed as mean and range of determinations made on duplicate cultures grown under the same conditions. In some experiments, an aliquot was applied to a G-50 Sephadex column (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) and the material eluted in the void volume was incubated with 10 U of *Streptomyces hyaluronidase* at 55°C for 20 h. The postdigestion sample was resubjected to Sephadex chromatography using a pyridine acetate buffer pH 5.8. The digested material, eluted after the void volume, was defined as HA. Recovery from chromatography was 95%.

**Cell-free hyaluronate synthetase assay.** Cultures were grown as described above except that 100-mm Diam plates were used. After 3 d of incubation in media supplemented with D-FCS, D-FCS +  $T_3$  or FCS, cell layers were washed two times with Hanks' balanced salt solution, then scraped

off the plates and concentrated by centrifugation. Following two washes with a buffer containing 4 mM dithiothreitol, 200 mM sucrose, 40 mM HEPES, pH 7.1, cells were disrupted by sonication (microtip at 100 W output for 1 s, repeated twice) on ice, and an aliquot was taken for protein determination by the method of Bradford (23). To assay hyaluronate synthetase activity, an aliquot of the sonicate was incubated with 0.32 μmol  $MgCl_2$ , 16 μmol sucrose, 0.63 μmol UDP-N-acetyl glucosamine (UDP-GlcNAc), 3 nmol UDP-glucuronic acid (UDP-GlcUA), 500,000 dpm UDP- $[^{14}C]GlcUA$ , and 3.2 μmol HEPES pH 7.1 in a total volume of 100 μl for 1 h at 37°C. The reaction was terminated by boiling for 5 min followed by centrifugation at 9,000 g. Aliquots of the supernatant were subjected to G-50 Sephadex chromatography and eluted with 0.5 M NaCl. Fractions were counted for radioactivity.

## RESULTS

Fig. 1 contains data concerning the reproducibility of the observed inhibitory effect of  $T_3$  on the incorporation of  $[^3H]$ acetate into GAG. The experiments labeled as B, C, and D were carried out at different times, over a 5-mo period, using cultures from the same donor and same batch of Tx-BS. Despite a two-

<sup>2</sup> We have previously shown that >95% of the radioactive material was degraded by hyaluronidase, chondroitinase ABC, and nitrous acid, as expected for GAG (19). In early experiments, postdialysis samples were analyzed for total uronic acid content by the method of Dische (22). Recovery was uniform.

fold variation in the absolute amount of [ $^3\text{H}$ ]GAG accumulation, the proportional decline as a result of  $\text{T}_3$  addition ranged from 39.5 to 60.3% and did not correlate with the absolute magnitude of [ $^3\text{H}$ ]acetate incorporation. The figure also shows that the inhibitory effect of  $\text{T}_3$  was reproduced when the hormone was added to either of the two types of hormone-depleted sera, D-FCS or Tx-BS (Fig. 1A and B).

To determine whether the inhibitory effect of  $\text{T}_3$  on [ $^3\text{H}$ ]acetate incorporation could be due to changes in the saturable uptake of acetate, unlabeled acetate was added in increasing concentrations (0, 100, 500, 2,500  $\mu\text{M}$ ) together with the labeled tracer (5  $\mu\text{M}$ ). As shown in Fig. 2, despite a 80% decrease in [ $^3\text{H}$ ]acetate incorporation caused by the decrease in specific activity, there were no changes in the proportion of inhibition by  $\text{T}_3$ . The latter ranged from 34.9 to 39.5%. The decrease in the specific activity of [ $^3\text{H}$ ]acetate was associated with a decrease in the uptake of [ $^3\text{H}$ ]acetate measured in aliquots of the washed cell layer. Uptake was 4.15, 3.40, 2.50, and 1.05% in the presence of 0, 100, 500, and 2,500  $\mu\text{M}$  of unlabeled acetate, respectively. Addition of  $\text{T}_3$  did not affect the uptake of the tracer. Furthermore, using fibroblasts from the same donor,  $\text{T}_3$  induced a 32.1% inhibition of [ $^3\text{H}$ ]glucosamine incorporation into GAG (Fig. 2).

To assess whether the observed effect of  $\text{T}_3$  on GAG accumulation is specific rather than a generalized inhibitory action of the hormone, the incorporation of [ $^3\text{H}$ ]leucine into proteins was examined under identical

experimental conditions. Results are given as mean  $\pm$  SD for duplicate cultures exposed to media supplemented with Tx-BS and TxBS +  $\text{T}_3$  (0.1  $\mu\text{M}$ ), respectively, for the period of 3 d. [ $^3\text{H}$ ]Leucine was added 24 h before harvesting. For fibroblasts exposed to Tx-BS and Tx-BS +  $\text{T}_3$ , respectively, retention of the isotope in the cell layer was  $5.0 \pm 0.1\%$  and  $4.3 \pm 0.2\%$ , and  $65.5 \pm 4.0\%$  and  $82.3 \pm 2.2\%$  of the  $^3\text{H}$  activity in cells was TCA precipitable. The total TCA-precipitable activity in cultures exposed to thyroid hormone-depleted and  $\text{T}_3$ -supplemented media was not significantly different, namely,  $3.78 \pm 0.26\%$  and  $4.17 \pm 0.08\%$  of the total radioactivity added. In both conditions, 87% of the TCA-precipitable radioactivity was recovered from the cell layer. After digestion with pronase,  $0.24 \pm 0.01\%$  and  $0.27 \pm 0.2\%$  of the radioactivity was TCA precipitable and only  $0.10 \pm 0.02\%$  and  $0.07 \pm 0.01\%$  of the activity remained in the TCA nonprecipitable, nondialyzable GAG-containing material.

The preceding experiments indicated that the inhibitory effect of thyroid hormone on [ $^3\text{H}$ ]GAG accumulation could not be accounted for on the basis of gross shifts in precursor pool size. Furthermore, the effect seemed to be somewhat specific in that similar changes in total cellular protein synthesis were not demonstrated. The issue of whether the observed effects of  $\text{T}_3$  on [ $^3\text{H}$ ]GAG accumulation were a result of changes in the rate of GAG degradation was addressed in a pulse-chase experiment. Cultures were incubated in media supplemented with D-FCS without or with

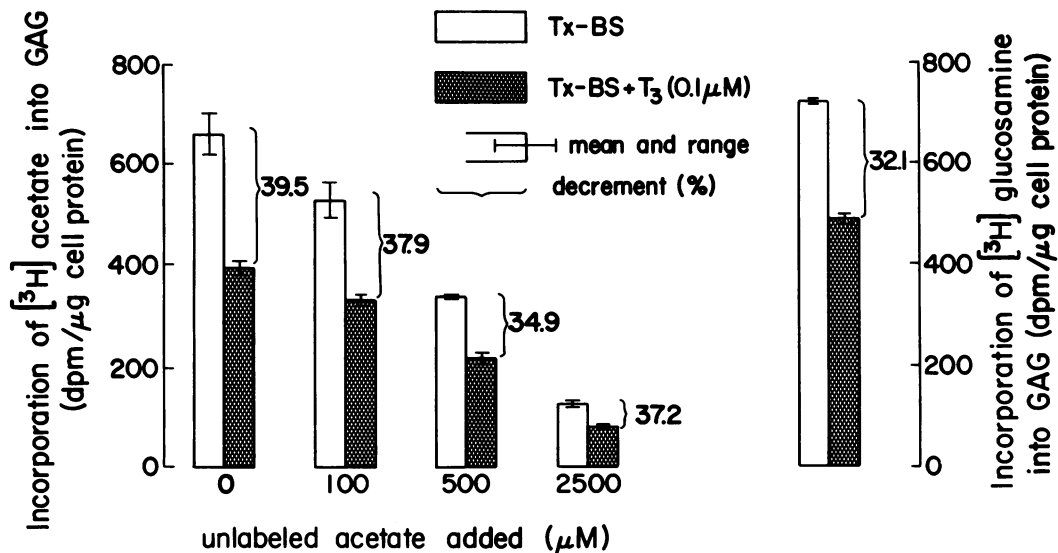


FIGURE 2 Effect of  $\text{T}_3$  on the incorporation of [ $^3\text{H}$ ]acetate and [ $^3\text{H}$ ]glucosamine into GAG. Fibroblasts obtained from the same subject were used in both experiments and were exposed to Tx-BS or Tx-BS +  $\text{T}_3$  for 3 d. Addition of unlabeled acetate decreased by 80% the incorporation of [ $^3\text{H}$ ]acetate into GAG but did not alter the inhibitory effect of  $\text{T}_3$ . The inhibitory effect on GAG was observed when both labeled acetate and glucosamine were used as substrates.

TABLE II  
Effect of  $T_3$  on [ $^3H$ ]GAG and [ $^3H$ ]HA Degradation in a Pulse-chase Experiment

	Duration of chase (hours)				
	0	12	20	36	63
[ $^3H$ ]GAG $\left(\frac{+T_3}{-T_3}\right)$	0.59	0.64	0.61	0.62	0.65
[ $^3H$ ]HA $\left(\frac{+T_3}{-T_3}\right)$	0.58	0.62	0.56	0.58	0.61
Proportion of [ $^3H$ ]HA in [ $^3H$ ]GAG (%)					
+ $T_3$	77.5	77.2	76.8	76.7	77.2
- $T_3$	79.7	81.5	83.8	82.4	82.1

Cultures were incubated either in D-FCS-containing medium ( $-T_3$ ) or in the same medium supplemented with  $0.1 \mu M T_3$  ( $+T_3$ ) for 48 h before pulse. The cell layer and media were analyzed for [ $^3H$ ]GAG and [ $^3H$ ]HA. Data are expressed as a ratio  $+T_3/-T_3$  for total [ $^3H$ ]GAG or [ $^3H$ ]HA following the chase. For time 0 (before chase), the results are derived from the cell layer analysis alone. The proportion of [ $^3H$ ]HA in [ $^3H$ ]GAG is also given as a percent.

$0.1 \mu M T_3$  for 48 h and then labeled for an additional 12 h with [ $^3H$ ]acetate. The chase was carried out for 12, 20, 36, and 63 h. As shown in Table II, total [ $^3H$ ]GAG and [ $^3H$ ]HA content, expressed as ratio in cultures exposed to  $T_3$  to corresponding cultures deprived of the hormone, did not change during the entire period of the chase. This result suggests that the rate of degradation of presynthesized GAG or HA is not affected by  $T_3$ . Furthermore, the proportion of [ $^3H$ ]HA in [ $^3H$ ]GAG was similar in fibroblasts exposed to  $T_3$  and deprived of  $T_3$ .

Since the rate of GAG degradation could not be implicated in the  $T_3$ -induced inhibition of [ $^3H$ ]GAG accumulation, an effort was made to directly assess the rate of synthesis utilizing a cell-free hyaluronate synthetase assay. When cell sonicates from cultures deprived of thyroid hormone were incubated with UDP-[ $^{14}C$ ]GlcUA,  $1,290 \pm 62$  ( $n = 3$ ) dpm/mg protein was incorporated into macromolecules compared with  $878 \pm 27$  dpm/mg protein incorporation in sonicates of  $T_3$ -repleted cells ( $P < 0.005$ ). 85–90% of the product was digested with *Streptomyces* hyaluronidase. Thus, cultures maintained in D-FCS +  $T_3$  have 32% less hyaluronate synthetase activity, a proportionally similar  $T_3$  effect to the [ $^3H$ ]acetate incorporation into GAG in a parallel experiment.

To assess whether the effect of thyroid hormone deficiency was reversible, several plates of fibroblasts from one subject were exposed to the D-FCS-supplemented medium for 10 d and then some were replenished with  $0.1 \mu M T_3$ . At various times, cultures were labeled for 24 h, and then harvested and analyzed for [ $^3H$ ]GAG content. Within 24 h of  $T_3$  addition, a decrease in [ $^3H$ ]GAG accumulation was evident compared with control cultures maintained in the  $T_3$ -de-

prived medium (Fig. 3). This reversal was maximal by 72 h. Because a maximal effect was noted within 72 h of culture in the thyroid hormone-deficient medium, most experiments were conducted after exposure to the hormone-deficient medium, (D-FCS or Tx-BS) for 3 d.

Various amounts of  $T_3$  were added to cultures incubated in D-FCS-supplemented medium (Fig. 4). The greatest amount of [ $^3H$ ]GAG accumulation occurred at the lowest concentration of  $T_3$  ( $0.06 \text{ nM}$ ), which represents residual hormone after resin treatment of the FCS. 73% of the maximal inhibitory effect of  $T_3$  was observed at a concentration of  $0.16 \text{ nM}$ , which was similar to that found in media supple-

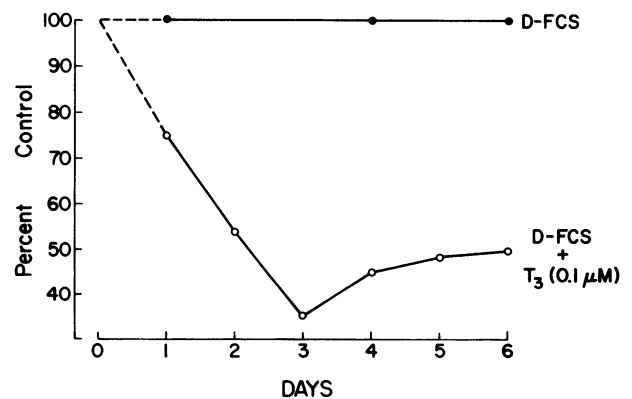


FIGURE 3 Time course of reversibility of the effect of thyroid hormone deficiency on [ $^3H$ ]acetate incorporation into GAG. Confluent cultures were incubated for 10 d in D-FCS. From time 0,  $0.1 \mu M T_3$  was added to some cultures (○), while others were continually maintained in D-FCS (●). Data from cultures with  $T_3$  added back are expressed as a percentage of control cultures that remained in D-FCS.

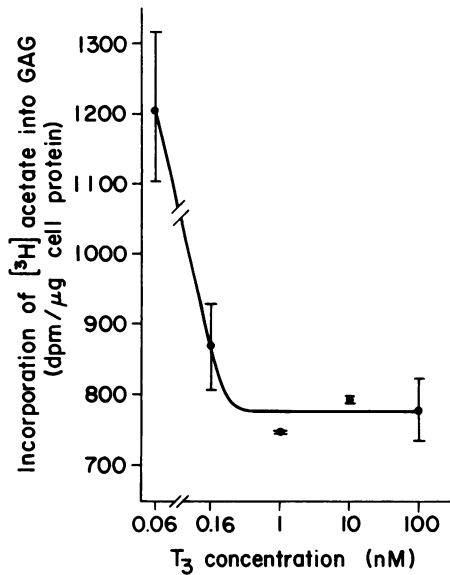


FIGURE 4 Effect of  $T_3$  concentration on [ $^3H$ ]GAG accumulation. Following confluence, cultures were incubated for 3 d in a D-FCS-containing medium in the presence of various concentrations of  $T_3$ . The  $T_3$  concentration of 0.06 nM represents the contribution of the  $T_3$  in the D-FCS and, further increments from 0.1 to 100 nM,  $T_3$  added to the medium. Data are expressed as the mean and range of determinations in duplicate cultures.

mented with untreated FCS. The addition of  $T_3$  in concentrations from 1 to 100 nM resulted in no further inhibition. Similarly, the addition of up to 100 nM  $T_3$  to cultures maintained in FCS resulted in no consistent change.

Fig. 5 contains data from an experiment involving skin fibroblasts from four individuals. Following confluence, cultures were exposed for 3 d to media supplemented with 10% Tx-BS without or with 0.1  $\mu$ M  $T_3$ . 24 h before harvesting [ $^3H$ ]acetate was added. Quantitation of [ $^3H$ ]acetate incorporation into macromolecules revealed that cultures incubated in the  $T_3$ -supplemented medium accumulated 27.8–53.2% less [ $^3H$ ]GAG than did twin sister cultures deprived of  $T_3$ . Despite variation in the absolute amount of [ $^3H$ ]GAG accumulation between subjects, the decline as a result of  $T_3$  addition invariably occurred. Because the protein per cell ratios remained constant in the two incubation conditions, the results are similar whether expressed per microgram cell protein or per cell. The cultures were of similar density when harvested.

## DISCUSSION

GAG metabolism has been studied in *in vitro* systems as a function of development (24), hormone action (25), cell density and transformation (26), and has been

correlated with such cellular metabolic events as cyclic AMP accumulation (27). The biological role of these complex sugars, however, has yet to be fully understood. There is evidence that they are important in cell attachment to substrate, communication with other cells, and, perhaps, insulate cells from chemical perturbations in the surrounding milieu (28).

Human skin fibroblasts are known to elaborate large amounts of GAG, most of which is HA, with considerably smaller amounts of chondroitin sulfate, heparan sulfate, and dermatan sulfate (26). A large proportion of HA synthesized in the cells is extruded into the medium (26, 29). Many aspects of HA synthesis are not well understood. Chain elongation, by sequential transfer of GlcUA and GlcNAc from their UDP donors, is independent of protein synthesis. Human skin fibroblast hyaluronidase has not been identified (30). This is not true of some animal fibroblast systems (31).

Our interest in the effect of thyroid hormone on GAG was prompted by *in vivo* observations (3, 4) and by our early work showing an inhibitory effect of  $T_3$  on [ $^3H$ ]GAG accumulation in fibroblasts from a single human donor (19). The present work contains data which indicate that earlier observations of inhibition of [ $^3H$ ]acetate incorporation into GAG by  $T_3$  is most likely due to a decrease in GAG synthesis. Indeed, we have shown that changes in the specific activity of added acetate that cause an 80% inhibition in the cell uptake of [ $^3H$ ]acetate and its incorporation into GAG, does not obliterate the effect of  $T_3$ . A similar inhibiting effect of  $T_3$  was obtained using [ $^3H$ ]glucosamine as a

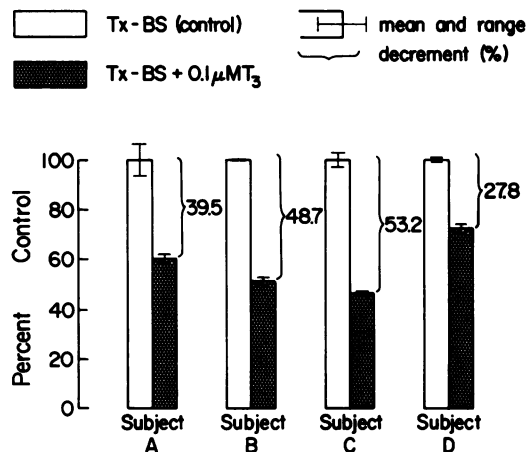


FIGURE 5 Effect of thyroid hormone on [ $^3H$ ]acetate incorporation into GAG in fibroblast cultures from four subjects. Cultures were grown to confluence in medium supplemented with FCS (Methods). Cultures were then shifted to a Tx-Bs-containing medium, without or with 0.1  $\mu$ M  $T_3$  for an additional 3 d of culture. The  $T_3$ -induced suppression [ $^3H$ ]acetate incorporation into GAG is indicated in percent  $\pm$  range of the untreated controls.

substrate. Under identical experimental conditions,  $T_3$  had no effect on total protein synthesis as assessed by the incorporation of [ $^3H$ ]leucine into TCA-precipitable material. Results of the pulse-chase experiment suggest that the observed effects of  $T_3$  cannot be explained on the basis of an enhanced rate of GAG degradation. Further, the data suggest that no significant amount of degradation of hyaluronidase-sensitive material has occurred over the course of the incubation, in keeping with a previous failure to demonstrate hyaluronidase activity in human skin fibroblasts (30). These observations, together with the results obtained with the cell-free hyaluronate synthetase assay, strongly suggest an inhibitory effect of thyroid hormone on HA synthesis. It is unclear whether  $T_3$  affects chain elongation or *de novo* synthesis. Further, it is not possible to determine whether the decreased synthetase activity is due to decreased or altered enzymes. The results do imply that some factor in polysaccharide synthesis is altered.

The inhibitory effect of thyroid hormone on GAG synthesis was reproducible using cultures from the same subject, and was present in fibroblast cultures from four other normal individuals. The dose range of  $T_3$  which inhibits GAG synthesis was similar to that stimulating cytoplasmic growth hormone mRNA accumulation and growth hormone synthesis in rat pituitary cells (32, 33). Changes were observed between the subphysiological levels of  $T_3$  (0.4 and 0.5 pM of free  $T_3$ ) and the physiological range of concentrations (1.6–2.1 pM of free  $T_3$ ). The effect is due to  $T_3$  rather than to other serum components, since it was observed in the presence of normal FCS depleted of thyroid hormone by resin treatment as well as with untreated BS obtained from a thyroidectomized animal. The relatively rapid reversibility of the effect of hormone deficiency and lack of effect on total protein synthesis suggest that, under the culture conditions chosen, thyroid hormone absence per se does not have generalized "toxic effects". Because hormone perturbations were made only after confluence and contact inhibition was achieved in normal growth conditions, the effects seen are those on a resting, nondividing population of cells. This strategy allows the assessment of nongrowth-related hormone responses. Since GAG accumulation per cell is known to vary with the phase of growth to confluence (26), uniformity in culture density is essential if conclusions are to be drawn regarding a primary hormone effect on GAG.

Estrogen and testosterone, as well as thyroid hormone, have been shown to have a variety of effects on GAG accumulation in different tissues (4, 34–37). Thyroid hormone appears to enhance the incorporation of [ $^{35}S$ ]sulfate into chondroitin sulfate in chick embryo costal cartilage (25). Schiller et al. (37), demonstrated

that thyroid hormone depletion resulted in an increased HA content in the skin of the rat. The findings *in vitro* are consistent with previously reported effects *in vivo*.

The finding of thyroid hormone inhibition of GAG synthesis prompts some speculations as to what effects, if any, the hormone may have upon other macromolecular metabolism. Preliminary work in this laboratory suggests that collagen accumulation may also be stimulated under  $T_3$ -deprived conditions (unpublished observations). These effects are perhaps related to common pool shifts or possibly to a coordinate shift in cellular energy expenditure in the compromised cell. Fibroblasts obtained from patients with Marfan's syndrome are also known to elaborate greatly increased amounts of HA when compared with normal controls (29). The increase appears to be due to enhanced rates of synthesis (38) analogous to the situation depicted here. The notion that a common mechanism for the two different entities exists is an intriguing one.

The present data suggest that thyroid hormone regulates GAG synthesis and accumulation in a dose- and time-dependent manner *in vitro* consistent with previously reported thyroid-sensitive metabolic events. Casting this hormone in the role of an inhibitor is somewhat contrary to the pervasive attitude that it is a stimulator. Recent reports that erythrocyte membrane  $Ca^{++}$ -ATPase activity from mature rats is inhibited by  $T_3$  (39) and that thyroid hormone both enhances and inhibits pretranslational events (40), together with the results reported here, shed new light upon the complexities of hormone-regulated gene expression in mammalian cells.

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